Evaluating the efficacy of LaSota vaccination induced protection in chickens upon challenge with a genotype IV strain of Newcastle disease virus

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Abstract Newcastle disease (ND) is a major risk to the poultry industry which results in severe economic loss throughout the world even with vaccination. The vaccine viruses that are used in many countries include the LaSota and other live viruses that were isolated in the early and late 1950s. Reports from several laboratories including ours indicate a greater variance of the circulating strains and recent classification indicates the existence of XVIII different genotypes of NDV strains. The efficiency of the LaSota vaccination in inducing protective immunity to different heterologous strains has been a question and its efficacy upon exposure to a virulent genotype IV strain has not been reported after 1989 world-wide except for India. Serum antibody negative (SAN) chicks of either sex obtained by hatching specific-pathogen-free (SPF) eggs were vaccinated with increasing doses of the vaccine virus from 10^1 to 10^7 EID₅₀ per bird delivered through oculo-nasal route and challenged 20 days later with NDV-2K3 (genotype IV) strain of virus isolated in the year 2000 from pigeon in India. The birds were monitored for serum antibody titers and following challenge for morbidity,

mortality, viral load in the cloacal swab and different tissues. We could clearly show that a minimum vaccine titre of 10^4 EID₅₀ could establish protective antibody levels and also prevent viral replication post challenge upon exposure to the virulent genotype IV strain. We conclude based on our results and previous observation that there do exist differences in the levels of the antibody that could limit viral replication and shedding upon exposure to different heterologous genotype of NDV. Developing a strain matched vaccine might less potential to result in better protection by limiting the viral shedding.

Keywords Viral shedding · Genotype II · Heterologous protection

Introduction

Newcastle disease (ND), an enzootic and contagious disease of poultry except in few countries, is caused by an avian paramyxovirus 1 (APMV1) of the *Avulavirus* genus in the subfamily of *Paramyxovirinae*. The virus is known to infect almost more than 241 species of birds [11] exhibiting a key feature of differential virulence depending on the pathotype of the virus and the species it infects. The major contributor to this feature is the formation of an active fusion (F) protein upon cleavage of a precursor and the number of basic amino acids at this cleavage site termed the fusion protein cleavage site (FPCS) [19, 21]. The combination of the amino acids and the distribution of the basic amino acids have been used to pathotype the virus as lentogenic, mesogenic or velogenic [5, 22]. In addition, to initiate an infection, the F protein along with haemagglutinin-neuraminidase (HN) protein enables the ND virus to bind and fuse the host cell membranes. Thus, following

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vaccination of chicken, antibodies to F and also HN are the major and primary component to result in vaccine induced protection [23].

It is known that antigenic and genetic diversity exists within the NDV isolates [2]. The sequence motifs around the FPCS and also the whole genome sequence have been used to group the circulating virus strains into eighteen different genotypes albeit they belong to APMV-1 serotype [7, 24]. It is interesting to note that the first panzootic of ND before 1960 was reported to involve genotypes II, III and IV. Thus the genotype IV virus has also been considered as the ancient genotype for the evolution of the current and commonly used genotype I (QV4, Ulster) and genotype II (Lasota, B1) vaccine strains globally [3, 12, 18] and the genotype IV viruses have not being reported from any part of the globe after 1980s. The predominant genotype circulating worldwide are V, VI and VII with the genotype VII being associated with many of the recent outbreaks in Asia [13, 14]. It is unique to note that the circulating genotypes belong to II, VII and XIII with genotype IV strain exists only in India [9, 25]. Several reports indicate that, usage of greater divergent strains as vaccine strains may not be a better choice in the control of ND as they do not reduce the virus shedding and transmission. There have also been reports on the ability of genotype II vaccine strains to completely protect against morbidity and mortality including the shedding of the challenge virus (genotypes II, VI, VII and IX) [6, 10, 16]. Even though studies have been made to determine the vaccine efficacy with the different genotypes, the ancient and virulent among the first panzootic are the genotype IV strains against which the effectiveness could not be determined as they have been considered as 'died-out' due to its non-existence worldwide.

Under Indian context, exploring the effectiveness of one of the widely used, current vaccine against the genotype IV challenge strain will be interesting. Hence, in this study we immunized groups of ND antibody free chicken (hatched out from SPF eggs) with different doses of genotype II vaccine strain (Lasota) and challenged them with genotype IV strain of velogenic virulent NDV (vvNDV) isolated from pigeon [25]. We monitored the immune response pre and post-challenge (PC) and also the virus shedding in immunized birds to evaluate the protective efficiency of the currently used genotype II commercial vaccine.

Materials and methods

Birds and viruses

Serum antibody negative (SAN) chicken of either sex was obtained by hatching specific-pathogen-free (SPF) eggs procured from M/s Venky's (India) Limited, SPF Division, Pune, Maharashtra, India at the Poultry Research Station

unit of Tamil Nadu Veterinary and Animal Sciences University, Chennai-51. The birds were maintained as per standards (RSPCA standards, April 2011) with provision of lighting for 23 h per day for the first week and reduced to 15–16 h. by 3 weeks to the end of the study. Pre-vaccination sera were collected from 25% of the birds used for the study at random on third day following hatch to confirm the absence of NDV antibodies. The birds were vaccinated with live lyophilized NDV (LaSota Strain) from Hester BP (Vet) on day 5 via ocularo-nasal route and challenged on twenty-first day post-vaccination (PV) via ocularo-nasal route with a velogenic strain of NDV (NDV-2K3/Pi/2000/IND—GenBank Acc. No. FJ986192) maintained at the virus repository of Department of Animal Biotechnology, Madras Veterinary College, Chennai, India.

Experimental design

At 5 days post hatch, the birds were arbitrarily divided into eight treatment groups: Group 1—mock vaccinated (vaccine diluent); Group 2 to Group 8—increasing doses of the vaccine virus from 10^1 to 10^7 EID₅₀ (in 100 µL) per bird delivered through ocularo-nasal route. Groups 1 and 2 contained 8 birds each while Groups 3 to 8 had 10 birds per group. The birds were monitored for clinical signs of vaccine reaction and were bled at weekly intervals to monitor their antibody status. Cloacal swabs were collected from all the birds prior to challenge. On day 21 post-vaccination, all the birds in groups 1 to 8 were challenged with the velogenic strain (NDV2K3) at a target titer of 10^5 EID₅₀ per bird delivered evenly between nasal and eye-drop routes. Birds were monitored for clinical signs of ND and mortality (birds with severe clinical signs were euthanized to collect the tissues). Cloacal swabs were collected on days 3, 6 and 9 post-challenge and also the tissues (spleen, proventriculus, caecal tonsil and brain) from the dead birds to determine the virus load and also for RNA isolation.

Serum NDV antibody titers

Serum samples collected in the study were tested for antibodies against NDV by haemagglutination inhibition (HI) test and also with a commercial ELISA kit (NDV Antibody Test Kit-CK116NDV, BioChek, ME). HI test was performed as per standard protocols and ELISA was performed as per manufacturer's instructions.

Virus titration

Titration in chicken embryo fibroblast cells

Cloacal swabs collected were dispersed in sterile saline containing antibiotics (100 IU/mL of penicillin, 1 mg/mL

of Streptomycin and 200 µg/mL Gentamycin sulphate). Following suspension, they were held at 4 °C for an hour, centrifuged at 5000 rpm for 10 min and the supernatant was used for titration. For the suspected tissues, a 1% tissue suspension of the organs collected post challenge from the different groups were prepared in sterile saline with antibiotics, held at 4 °C for an hour, centrifuged at 5000 rpm for 10 min and the supernatant used for titration. Titration was performed in chicken embryo fibroblast (CEF) cells prepared from 7 to 9-day old SPF embryonated chicken eggs as per standard protocols. Virus titres (TCID₅₀) in the samples were determined by the method of Reed and Muench (1938).

Determination of virus load by qRT-PCR

Viral nucleic acid was extracted from the cloacal swabs and tissue suspensions by using Viral RNA extraction Kit (Roche, India) as per the manufacturer's instructions, converted into cDNA using High Capacity cDNA Synthesis kit (Applied Biosystems, India) and the NDV load in different samples were determined using qRT-PCR employing M gene specific (MF: 5'-ATCACCACC-TATGGATTC-3'; MR: 5'-AGTATTAGTTGCACTCTT-3') primers in a SBYR green based assay. To convert the obtained Ct value into virus titres, we developed a standard curve with qRT-PCR performed on RNA extracted from known EID₅₀ titres of commercial vaccine virus. The virus load in the cloacal swabs and the tissue suspensions were expressed as EID₅₀ using the above standard curve.

Statistical analysis

The mean values for antibody titres and virus load assessed by different methods for the different groups were compared by ANOVA with Bonferroni's test for multiple comparisons employing GraphPad prism software. The differences among means were considered significant at $p < 0.05$.

Results

Serum antibody titers

In this study, we maintained groups of chickens with varying levels of antibodies to NDV by immunizing them with different titres of vaccine virus (10^1 – 10^7 EID₅₀) and then challenged them with a heterogenic heterologous NDV (genotype IV), 21 days post vaccination. Serum samples collected pre-immunization, 19 days post vaccination (PV) and 3, 6 and 9 days post challenges (PC) were tested for antibodies to NDV.

The mean pre-immunization HI antibody titre was found to be 4.66 ± 2 (Table 1). At 19 days PV all the birds in the Group 8 (10^7 EID₅₀) to Group 5 (10^4 EID₅₀) seroconverted with statistically significant increased ($p < 0.05$) serum HI antibody titres that were protective (HI titre of ≥ 32). However, the birds in group 4 (10^3 EID₅₀) and group 3 (10^2 EID₅₀) which had a lower pre-challenge titres on day 19, developed protective titres of 88 ± 48 and 112 ± 32 respectively ($p < 0.05$) at day 3 PC (Table 1). The birds in group 1 and group 2 did not have any detectable levels of antibody even post-challenge probably due to very low viral load in the vaccine to induce an effective immune response and also due to the shorter time between the challenge and death of the birds. The antibody titres were monitored pre-immunization; 19 days post vaccination and days 3, 6 and 9 post-challenge by BioChek ELISA kit. On day 19 PV all the birds in the group 8 (10^7 EID₅₀) to group 5 (10^3 EID₅₀) sero-converted with statistically significant increase in serum ELISA titres pre-challenge (titre of ≥ 1159). The birds in group 4 (10^3 EID₅₀) and group 3 (10^2 EID₅₀) even though had lower titres PV, developed significantly increased titres (titre of ≥ 1159) at day 3 and 6 PC respectively (Table 2). Anti-NDV antibodies could not be detected by both by HI and ELISA in the group 1 and 2 post challenge.

Morbidity and mortality

Prior to immunization and challenge, there were no clinical signs of NDV in the different groups. Following challenge all the birds in the groups 1 (mock control) and 2 (10^1 EID₅₀) exhibited severe conjunctivitis and depression 5–6 days post challenge and 100% mortality by day 14 post challenge. The birds in the group III (10^2 EID₅₀) exhibited mild depression and conjunctivitis at 4–5 days post challenge but showed no clinical signs afterwards and no mortality was observed (Fig. 1).

Virus shedding

Cloacal swabs were collected on day 19 PV, days 1, 3, 6 and 9 PC from the different groups. Birds in the different groups revealed a lower viral load of 2.24×10^2 TCID₅₀ until day 1 PC. The viral shedding was higher with a load of $4.39 \pm 2.15 \times 10^2$ TCID₅₀ in group 3 (10^2 EID₅₀) on day 3 PC which decreased to 2.24×10^2 TCID₅₀ on day 6 PC. There was a significantly higher shedding of the virus in the group 2 and group 1 (mock control) on day 3 post-challenge ($1.69 \pm 1.90 \times 10^5$ TCID₅₀) and the birds started exhibiting clinical symptoms of ND. The shedding correlated with the higher virus load in different organs (spleen, proventriculus, caecal tonsil and brain) in group 2 and group 1 with very low levels in the group 3. The details on the virus load

Table 1 Serum NDV antibody titres determined haemagglutination inhibition test

Treatment group	No. pre/post challenge (9 PC)	Pre vaccinated	HI titres $\pm$ SE			
			19 days PV	3 days PC	6 days PC	9 days PC
Group 8 [10^7 EID ₅₀]	8/8	4.66 $\pm$ 2	<i>104 $\pm$ 36.2</i>	384 $\pm$ 52.3	96 $\pm$ 13.1	56 $\pm$ 5.66
Group 7 [10^6 EID ₅₀]	8/8		<i>112 $\pm$ 11.3</i>	416 $\pm$ 144.9	96 $\pm$ 13.1	96 $\pm$ 13.1
Group 6 [10^5 EID ₅₀]	8/8		<i>56 $\pm$ 15.2</i>	144 $\pm$ 25.3 ^{ab}	44 $\pm$ 7.59	18 $\pm$ 3.2
Group 5 [10^4 EID ₅₀]	8/8		<i>136 $\pm$ 29.1</i>	384 $\pm$ 46.7 ^c	56 $\pm$ 5.1	40 $\pm$ 8.8
Group 4 [10^3 EID ₅₀]	8/8		28 $\pm$ 2.5	88 $\pm$ 15.2 ^{abd}	64 $\pm$ 0	40 $\pm$ 5.1
Group 3 [10^2 EID ₅₀]	8/8		32 $\pm$ 7.6	92 $\pm$ 10.1 ^{abd}	160 $\pm$ 35.1	144 $\pm$ 40.9 ^c
Group 2 [10^1 EID ₅₀]	8/0		2 $\pm$ 2	0	0	0
Group 1 [unvaccinated]	8/0		2 $\pm$ 0	0	0	0

The HI titer results are expressed as mean $\pm$ standard error. Means within the same column with different superscripts (a, b, c, d with 10^7 , 10^6 , 10^5 , 10^4 treatment group respectively) are significantly different by two-way ANOVA and Bonferroni Posttest ($p < 0.05$). Unvacc—diluent used. (n = 10 birds per group except for group 1 and 2 with 8 birds)

Italics highlights the protective HI antibody titres in groups 5 to 8 and lower than the protective titres in groups 3 and 4 on 19 days PV. The titres reached protective levels in groups 3 and 4 on day 3 PC

PV post vaccination; PC post challenge

Table 2 Serum NDV antibody ELISA titres

Treatment group	No. pre/post challenge (9PC)	Pre vaccinated	ELISA titres $\pm$ SE			
			19 days PV	3 days PC	6 days PC	9 days PC
Group 8 [10^7 EID ₅₀]	8/8	0 $\pm$ 0	8830.7 $\pm$ 2741.5	6512.7 $\pm$ 936.7	8015.8 $\pm$ 804.5	5839.8 $\pm$ 1369.2
Group 7 [10^6 EID ₅₀]	8/8		12,393.0 $\pm$ 4111.5	8459.6 $\pm$ 3254.2	7992.7 $\pm$ 2598.1	7211.3 $\pm$ 2546.1
Group 6 [10^5 EID ₅₀]	8/8		5064.8 $\pm$ 1184.3 ^b	7396.0 $\pm$ 1434.9	6580.4 $\pm$ 1010.7	4760.2 $\pm$ 350.5
Group 5 [10^4 EID ₅₀]	8/8		7909.9 $\pm$ 1261.6	2404.1 $\pm$ 463.3 ^b	2302.9 $\pm$ 388.9 ^{ab}	3154.2 $\pm$ 302.8
Group 4 [10^3 EID ₅₀]	8/8		3224.1 $\pm$ 504.9 ^{ab}	1426.8 $\pm$ 283.5 ^{abc}	2489.9 $\pm$ 237.9 ^{ab}	1785.2 $\pm$ 410.2 ^b
Group 3 [10^2 EID ₅₀]	8/8		937.1 $\pm$ 189.01 ^{abd}	667.8 $\pm$ 158.2 ^{abc}	1251.3 $\pm$ 111.9 ^{ab}	903.5 $\pm$ 1970.1 ^{ab}
Group 2 [10^1 EID ₅₀]	8/0		n/a	n/a	n/a	n/a
Group 1 [unvaccinated]	8/0		n/a	n/a	n/a	n/a

The ELISA titer results are expressed as mean $\pm$ standard error. Titers of 828–1158 is considered suspected and >1158 is considered positive. Means within the same column with different superscripts (a, b, c, d with 10^7 , 10^6 , 10^5 , 10^4 treatment groups respectively) are significantly different by Two-way ANOVA and Bonferroni Posttest ($p < 0.05$). Unvacc—diluent used. (n = 10 birds per group except for group 1 and 2 with 8 birds)

Italics denotes that titres 1158 are considered positive which is observed in groups 5 to 8 on 19 days PV, while in groups 3 and 4 the ELISA antibody titres are positive only on days 3 and 6 PC, respectively

in the cloacal swabs and different organs are listed in Tables 3 and 4 respectively. Virus load in the cloacal swabs assessed by qRT-PCR (EID₅₀) also correlated with the results of the titration in CEF cells (Fig. 2c). Even though the virus titres in the different organs of group III were significantly higher on day 9 PC, the shedding was lower in the cloacal swabs (Fig. 2c, d) which can be attributed to the protective levels of antibodies on day 3 PC.

Discussion

In countries where ND is enzootic, vaccination is the major intervention to control the disease along with other bio-security measures. In recent years, there have been reports from different research groups indicating the contrast and greater variability between sero-group versus genotype in NDV [17]. Recent report also indicates the observed wider

Fig. 1 Percentage survival post NDV-2K3 challenge in the different groups. The chickens ($n = 8$) in each group (vaccinated different EID₅₀ titer of Lasota vaccine virus) were challenged at day 19 post-vaccination with 10^5 EID₅₀ of the NDV-2K3 genotype IV vaccine strain. Mortality was monitored for 14 days after challenge

host range; vaccine spurt or greater virulence might be contributed by the existence of genetically distant strains [1]. With increasing facilities to generate sequence data and effective analysis using bioinformatic tools, classification of the existing NDV isolates world-wide has resulted in the description of 18 different genotypes [24] with the circulating genotypes differing regionally [9, 25]. Vaccines strains like Lasota, used in different parts of the world have been derived from the actively circulating genotypes of the 1960s which exhibit greater variability with the current and existing strains circulating globally.

There exists almost 12–20% variability at the amino acid level between the vaccine strains in use and the circulating virulent field strains, raising a concern on the ability of the vaccine strains to provide ample protection against the novel field variants of NDV. This hypothesizes on the efficiency of vaccine strains like LaSota on different

circulating genotypes (genotypes II, III, VIg, VIb, VIId, and IX) of NDV [4, 6, 10, 12, 15, 17] with no report on the vaccine efficacy with a genotype IV virus, a classic example of an ancient virulent virus (e.g. Hertz 33; died out genotype globally since 1980s). This study also gains significance in the existence and circulation of this genotype only in India [9, 25, 26]. Comparison of the amino acid identities in the F and HN protein across LaSota (genotype II) and the NDV-2K3 (genotype IV) indicated a divergence of 10.5 and 13.2% respectively [25] and confirms the hypothesis that antigenic divergence across the vaccine and field ND virus could reduce the efficiency of vaccination [8]. We used serum antibody negative chicks in our study and generated experimental groups with varied antibody levels by vaccination with varied titres of the vaccine virus (ten-fold decreasing titres from 10^7 to 10^1 EID₅₀) and challenged them at 21 days with the genotype IV strain (NDV2K3). Proper vaccination and absence of maternal antibodies provides adequate protection against mortality and morbidity as well as shedding of the virus. This study also speculates that increasing the vaccine virus titer beyond a limit does not provide significant difference in the protection exhibited in the absence of maternal antibody.

With the varying titres of the Lasota vaccine, we could clearly show that a lower virus titre of 10^4 EID₅₀ resulted in significantly higher HI antibody titres (above the protective antibody titre of 32) at 19 days post vaccination (PV) when compared with the unvaccinated birds. Antibody titres measured by ELISA in these groups were also significantly higher which completely protected them from mortality and morbidity as well resulted in a significantly lower level of virus shed (2.24×10^2 TCID₅₀) in the cloacal swabs collected post challenge (PC) (with genotype IV). These birds also did not exhibit any viral load in the different organs tested. Vaccine virus dose of 10^3 and 10^2 EID₅₀ did

Table 3 Viral shedding in the cloacal swabs—titration in CEF (TCID₅₀)

Treatment group	19 Post vac.	20 Post vac.	3 Post chal.	6 Post chal.	9 Post chal.
Group 8 [10^7 EID ₅₀]	—	—	—	—	—
Group 7 [10^6 EID ₅₀]	—	—	—	—	—
Group 6 [10^5 EID ₅₀]	$4.20 \pm 2.17 \times 10^2$	2.24×10^2	—	—	—
Group 5 [10^4 EID ₅₀]	$4.73 \pm 1.57 \times 10^2$	2.24×10^2	—	—	—
Group 4 [10^3 EID ₅₀]	2.24×10^2	2.24×10^2	—	—	—
Group 3 [10^2 EID ₅₀]	2.24×10^2	2.24×10^2	$4.39 \pm 2.15 \times 10^2$	—	—
Group 2 [10^1 EID ₅₀]	—	—	$1.26 \pm 1.07 \times 10^5$ ^a	—	—
Group 1 [unvaccinated]	—	—	$1.69 \pm 1.90 \times 10^5$ ^a	—	—

The viral titers TCID₅₀ are expressed as mean $\pm$ standard error. Mean viral titer in 10^1 EID₅₀ and unvaccinated are significantly higher over the other groups as assessed by two way ANOVA and Bonferroni Posttest ($p < 0.001$). The cloacal swab samples from each bird were pooled to result in 3 samples per group and used for titration and thus the values represent the titers in the overall number of maintained in each group ($n = 10$ birds per group except for group 1 and 2 with 8 birds)

Italics denotes significantly higher viral titres in the cloacal swabs on day 3 PC

^aSignificantly higher viral titres in the cloacal swab in groups 1 and 2 on day 3 PC

Table 4 Viral load in the different organs—titration in CEF (TCID₅₀)

Treatment group	From tissue suspensions			
	Spleen	Proventriculus	Caecal tonsil	Brain
Group 8 [10 ⁷ EID ₅₀]	–	–	–	–
Group 7 [10 ⁶ EID ₅₀]	–	–	–	–
Group 6 [10 ⁵ EID ₅₀]	–	–	–	–
Group 5 [10 ⁴ EID ₅₀]	–	–	–	–
Group 4 [10 ³ EID ₅₀]	–	–	–	–
Group 3 [10 ² EID ₅₀]	2.24 × 10 ²	2.24 × 10 ²	2.24 × 10 ²	2.24 × 10 ²
Group 2 [10 ¹ EID ₅₀]	2.23 ± 1.86 × 10 ⁴	2.52 ± 2.74 × 10 ⁷ ^a	7.15 ± 1.67 × 10 ⁶	2.46 ± 3.07 × 10 ⁵
Group 1 [unvaccinated]	1.59 ± 2.72 × 10 ⁴	7.59 ± 2.3 × 10 ⁴ ^b	1.75 ± 2.66 × 10 ⁴	7.59 ± 2.3 × 10 ⁴

The viral titers TCID₅₀ are expressed as mean ± standard error. Mean viral titer in 10¹ EID₅₀ vaccinated and unvaccinated are significantly higher over the other groups as assessed by Two-way ANOVA and Bonferroni Posttest ($p < 0.001$). All the birds that were alive were euthanized at the end of the study for sample collection. The tissue samples from each bird were pooled to result in 3 samples per group for each tissue and used for titration and thus the values represent the titers in the overall number of maintained in each group ($n = 10$ birds per group except for group 1 and 2 with 8 birds)

Italics denotes significantly higher viral titres in the different organs in groups 1 and 2 PC

^a, ^bSignificantly higher viral titres in proventriculus than the other organs tested in groups 1 and 2

not result in protective HI titres at 19 days PV. However, PC these birds developed protective titres at 3 days PC which also increased on day 6 and 9 PC indicating replication of the challenge virus in these birds. This is very much evident from the significantly high virus titres in the cloacal swabs 3 days PC ($4.39 \pm 2.15 \times 10^2$ TCID₅₀) and lower loads of virus (2.24×10^2 TCID₅₀) in the different organs tested in the 10² EID₅₀ vaccine group.

Virus load in the cloacal swabs and the different organs (proventriculus, spleen, caecal tonsil and brain) tested, and the higher HI antibody titres PC in the 10³ and 10² EID₅₀ vaccinated groups indicate the need for at least 10⁴ EID₅₀ virus titre in the vaccine to protect the birds against the disease and also to prevent infection and replication of the virus when challenged with a genotype IV field virus (NDV-2K3). A report on LaSota vaccination failed to prevent viral replication at doses of 10³–10⁵ (EID₅₀/bird) even though it clinically protected the birds upon challenge with genotype VIIId virus and stressed the need for vaccine titres of 10⁶–10⁸ EID₅₀ to prevent the challenge virus replication [6]. Our observations in this study are in agreement to the fact that prevention of infection following challenge is mediated through the neutralizing antibodies generated [20, 27] and there do exist some differences in the levels of the antibody that provide protection against difference genotypes. With respect to genotype IV exposure it confirms that, a vaccine virus titre of less than 10⁴ EID₅₀ until 10² EID₅₀ resulted in lower shedding in the all birds and directly correlates with the increase in the antibody titres PC as well as protection from the disease. The observation on the protective antibody levels and the absence of viral replication/shedding in birds vaccinated

with 10⁴ EID₅₀ of vaccine virus indicate saturation of the mucosal surfaces thereby preventing infection. If this hypothesis is true, determining the “serum antibody titre threshold” that provides protection would be very much useful in the field upon exposure to even a different genotype of a field virus. Threshold factor is important as higher vaccine titres reflect on the vaccine cost and also to the greater cost due to the vaccine reaction in the birds (decreased growth rate and lower egg production). Our study on SPF birds provides the status upon exposure to a genotype IV NDV challenge and may not replicate the situation in the field as they are associated with other problems like the maternal antibodies, differences in immune response across batches and the vaccine application techniques.

Fig. 2 Determination of the viral load by quantitative RT-PCR in the cloacal swabs (c) and different organs (d) collected from the birds belonging to the different vaccinated groups. The viral load is expressed in terms of mean EID₅₀ values ± standard error. **a** Melt curve analysis of the products amplified from different viral loads of the vaccine strain that was used to generate the standard curve; **b** standard curve generated with different viral loads of the vaccine strains; **c** viral load (EID₅₀) in the cloacal swabs collected on day 19 PV and days 3, 6 and 9 post challenge; **d**, viral load in the different organs of the group 3 (day 9 PC) and the birds that died in the groups II and I (9 days PC). Note The low levels of virus in the groups 3, 4 and group 5 on day 9 PV. Even though the organs (proventriculus, caecum, spleen and brain) exhibited significant viral load in the group 3 the shedding of the virus in the cloacal swab were lower probably due to protective antibody titers on day PC. The cloacal swabs samples from each bird were pooled to result in 3 samples per group and used for titration and thus the values represent the titers in the overall number of maintained in each group ($n = 10$ birds per group except for group 1 and 2 with 8 birds)

Previous observations have confirmed the protection offered by LaSota vaccine strain to heterologous challenge of few genotypes [6, 10, 16]. This study adds on to the earlier observations to support the protective response of LaSota vaccination to an exposure to an ancient and died out divergent genotype of NDV strain (genotype IV). The study also stress the need for efficient delivery of the existing vaccines to develop sufficient post vaccinal antibody response to limit viral replication that do vary with exposure to a heterologous genotype of NDV and the existence of some differences in the levels of the antibody that provide protection against difference genotypes. In addition, it is also felt that efforts to develop a strain-matched vaccine strain might have less potential to result in better protection by decreasing the extent of viral shedding.

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